

Molecular Analysis of TCR and Peptide/MHC Interaction Using P18-I10-Derived Peptides with a Single D-Amino Acid Substitution

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ABSTRACT For the structural analysis of T-cell receptor (TCR) and peptide/MHC interaction, a series of peptides with a single amino acid substitution by a corresponding D-amino acid, having the same weight, size, and charge, within P18-I10 (aa318–327: RGPGRFVTL), an immunodominant epitope of HIV-1 IIIB envelope glycoprotein, restricted by the H-2D^d class I MHC molecule, has been synthesized. Using those peptides, we have observed that the replacement at positions 324F, 325V, 326T, and 327I with each corresponding D-amino acid induced marked reduction of the potency to sensitize targets for P18-I10-specific murine CD8⁺ cytotoxic T lymphocytes (CTLs), LINE-IIIB, recognition. To analyze further the role of amino acid at position 325, the most critical site for determining epitope specificity, we have developed a CTL line [LINE-IIIB(325D)] and its offspring clones specific for the epitope I-10(325v) having a D-valine (v) at position 325. Taking advantage of two distinct sets of CD8⁺ CTLs restricted by the same D^d, three-dimensional structural analysis on TCR and peptide/MHC complexes by molecular modeling was performed, which indicates that the critical amino acids within the TCRs for interacting with 325V or 325v appear to belong to the complementarity-determining region 1 but not to the complementarity-determining region 3 of V β chain.

INTRODUCTION

Immune responses to viral infection include both humoral and cell-mediated effector mechanisms. The major effector cells in cellular immunity are CD8 molecule-expressing cytotoxic T lymphocytes (CTLs) that can recognize and kill virus-infected cells. In general, endogenously synthesized antigens such as virus-derived proteins are fragmented inside of the cells and are presented on the cell in conjunction with class I major histocompatibility complex (MHC) molecules. Such processed epitope peptides associated with the class I MHC molecules can be recognized by CTLs via their specific T cell receptors (TCRs).

The TCRs expressed on the cell surface of T lymphocytes contain similar structural patterns with immunoglobulin-like domains, comprising one variable and one constant, as well as a transmembrane domain and a short cytoplasmic tail. The specificity for T-cell recognition seems to be determined by the variable domains, TCR V α and TCR V β , within two heterodimeric subsets, TCR α and TCR β . Several recent findings have indicated that the TCR α and β heterodimers are oriented to the long axis of the epitope-peptide/MHC complex (1), in which the V α domain appears to cover the amino-terminal half of the epitope peptide, whereas V β is located over the carboxyl-terminal portion of the epitope (2).

Among those variable V α and V β domains, three hypervariable complementarity-determining regions (CDRs), termed CDR1, CDR2, and CDR3, seem to directly interact with the peptide/MHC complex. Because the degree of variability is

the greatest in the CDR3 loop generally, and it is positioned more closely over the epitope peptide than other CDR1 and CDR2 loops, the antigen specificity has been considered to be associated with the CDR3 but not with CDR1 or CDR2, which were predicted to interact principally with the MHC molecules (3,4). Indeed, according to a recent report on the murine K^b class I MHC molecule-restricted epitope octapeptide (pKB1: KVITFDL) recognized by KB5-C20 TCR (5), TCR plasticity is primarily restricted to the CDR3 loops of the V β domain. Nevertheless, recent crystallographic analyses on various TCR and peptide/MHC interactions have suggested the possibility of direct contact for both CDR1 and CDR3 in the TCR α and TCR β chains with the antigenic peptide/MHC complex (5,6). Therefore, to understand more precise molecular interactions determining T-cell specificity through TCR-mediated peptide/MHC complex recognition, we took advantage of the following known materials to accomplish the analysis.

We have established CD8⁺, H-2D^d class I MHC molecule-restricted murine CTL line, LINE-IIIB, specific for the envelope glycoprotein 160 (gp160) composed of ~900 amino acids derived from one of the most commonly used IIIB strains of human immunodeficiency virus type-1 (HIV-1), a causative agent for acquired immunodeficiency syndrome (AIDS) (7). Then, we have identified an immunodominant epitope within the gp160 as a 15-residue peptide, P18IIIB (aa315–329: RIQRGPGRFVTIGK), for the LINE-IIIB recognition (7) as well as the minimal active 10-residue peptide, P18-I10 (aa318–327: RGPGRFVTL) within P18IIIB (8). Moreover, although the position of P18IIIB is located in the hypervariable portion (termed V3 domain) of the viral envelope, the site has turned out to be recognized by various isolate-specific CTLs in an isolate-specific manner (9,10), and

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a number of distinct class I MHC molecules did present the P18IIIB to each specific CTL (11). Furthermore, the P18IIIB in the V3-domain was found to be overlapped with the major determinant sites for neutralizing antibodies against HIV-1 in an isolate-specific manner (12–14) and also to be recognized by CD4-positive helper T lymphocytes specific for HIV-1 in a class II MHC molecule-restricted manner (8,15). In addition, human CTLs did see the P18IIIB when presented by HLA A2 and A3 (16). These findings indicate that the P18IIIB appears to be a highly attractive epitope for the development of peptide-based vaccine against AIDS, and thus, it is important to study the precise interaction between the epitope P18IIIB and their specific TCRs to study the manner of T-cell-mediated immune responses.

Using a series of peptides with an alanine (A) substitution at each position, we observed that amino acids at positions 322R and position 324F were critical for D^d binding and that position 325V within P18-II10 was essential for interacting with TCRs (17). Also, C-terminus 327I appears to be critical for D^d binding to form the D^d-binding motif (8,18). In addition, we found the HIV-MN isolate-specific CTLs also saw the corresponding minimal active site, MNT10 (aa318–327: IGPGRFYTT) in association with the same D^d molecules, and replacement of just a single residue, 325V with 325Y, within the P18-II10 or vice versa within the MNT10 was sufficient to reciprocally interchange the specificities for these two non-cross-reactive sets of CTLs (9,10). Thus, a single side chain at position 325 can play a critical role in determining the epitope specificity within both P18-II10 and MNT10 presented by the same class I MHC molecule D^d for CD8⁺ CTL TCR recognition mediated by the CDRs.

It has been reported that the charge of the amino acid might also affect interaction between TCRs and peptide/MHC complexes (19). Indeed, when negatively charged glutamic acid (E) at position 436 within HIV-1-envelope-derived helper T-cell epitope T1 (aa428–443; KQIINMWQEVG-KAMYA) (20) was substituted with either uncharged alanine (A) or size-conservative, uncharged glutamine (Q), stimulatory capacity of the substitute peptides for T1-specific T hybridomas was significantly enhanced, although charge-conservative aspartic acid (D) substitution did not show any enhancement (21). Moreover, substitution at position 6Q in the immunodominant CTL epitope for vesicular stomatitis virus (RGYVYQGL, VSV8) presented by K^d class I MHC molecules to a negatively charged residue such as 6E or 6D induced a change at position 93S of TCR CDR3 α to a positively charged residue 93R or 93K (22). Therefore, particularly to reduce the influence of charges of each amino acid as well as the size and molecular weight on TCR-mediated recognition, a series of P18-II10-derived peptides with a single amino acid substitution by D-amino acid at each corresponding site have been synthesized. Using those D-amino acid-substituted peptides, we found apparent reduction of specific cytotoxic activity in the 325V-specific LINE-IIIB cells by the replacement of 325V with D-type valine at position 325,

represented as I10(325v). Then, we attempted to establish CTL lines specific for the epitope I-10(325v) by immunization with dendritic cells pulsed with the peptide, I10(325v) (23). We have successfully generated both a 325(v)-specific CTL line and clones that did not cross-react with the original P18-II10.

Taking advantage of two distinct sets of CD8⁺ CTL clones specific for either P18-II10 bearing L-type valine or I10(325v) having D-type valine at position 325 presented by the same MHC molecules D^d, we attempted to study the three-dimensional (3D) structural analysis on TCRs and peptide/MHC complexes. Here, using molecular modeling analysis, we would like to show that the critical amino acids for interacting with P18-II10 in determining epitope specificity appear to be the peptide DMSHET within CDR1 of V β 7, whereas those for interacting with I10(325v) appear to be TNSHNY within CDR1 of V β 8.3.

MATERIALS AND METHODS

Mice

Female BALB/c (H-2^d) mice were purchased from Charles-River Japan Inc. (Tokyo, Japan). The mice were 6 to 10 weeks of age and were maintained in a specific-pathogen-free environment. All experiments were performed according to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

Synthetic peptides

Peptides were synthesized and purified as described previously (24). Table 1 summarizes the peptides used in this study; L-amino acids are represented as capital letters, and D-amino acids as lower-case letters.

Transfectants

BALB/c.3T3 (H-2^d) fibroblast transfectants expressing the HIV-1 gp160 of IIIB isolate (15–12) and control transfectants with selectable marker genes (Neo) (7, 17) were used for the CTL assay. Murine L-cells (H-2^b) transfected with H-2D^d (T4.8.3) (25), H-2L^d (T.1.1.1) (25), and H-2K^d (B4III2) (26) were used to determine the MHC class I restriction of the generated CTL line and clones.

Monoclonal antibodies

We used fluorescein isothiocyanate-conjugated antimouse CD3 (145-2C11), $\alpha\beta$ TCR (H57-597), CD4 (RM4-5), and CD8 (53-6.7) monoclonal antibodies (PharMingen, San Diego, CA) to determine the cell surface molecules of the established CTL lines and clones.

CTL lines and clones

The P18-II10-specific CTL line, LINE-IIIB, was generated as described previously (7). Based on a previously reported procedure (23), a CTL line specific for I10(325v) was generated from spleen cells of BALB/c mice immunized with I10(325v)-pulsed splenic dendritic cells. Briefly, immune spleen cells were restimulated in vitro with mitomycin C-treated I10(325v)-pulsed syngeneic BALB/c.3T3 fibroblasts in 24-well culture plates containing 1.5 ml of complete T-cell medium composed of RPMI 1640 medium

TABLE 1 Sequences of substituted peptides used in this study

Peptide	Sequence*															
Residue No.	315								325				329			
P18IIIB	R	I	Q	R	G	P	G	R	A	F	V	T	I	G	K	
P18-I10				R	G	P	G	R	A	F	V	T	I			
I10(325I)											I					
I10(325L)											L					
I10(325A)											A					
I10(325Y)											Y					
I10(325F)											F					
I10(325H)											H					
I10(325T)											T					
I10(325S)											S					
I10(325E)											E					
I10(325K)											K					
I10(325R)											R					
I10(325P)											P					
I10(318r)			r													
I10(320p)					p											
I10(322r)							r									
I10(323a)								a								
I10(324f)									f							
I10(325v)										v						
I10(326t)												t				
I10(327i)													i			
I10(325v)				R	G	P	G	R	A	F	v	T	I			
I10(325i)											i					
I10(325l)											l					
I10(325a)											a					
I10(325y)											y					
I10(325f)											f					
I10(325h)											h					
I10(325t)											t					

*Sequences of substituted peptides are derived from and aligned with the sequence of P18-I10, an immunodominant epitope of HIV-1 gp160 envelope glycoprotein of the IIIB strain for the H-2D^d-restricted murine CTL. In these peptides, L-amino acids are expressed as capital letters and corresponding D-amino acids are expressed as small letters.

supplemented with 2 mM L-glutamine, 50 μ M 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10% heat-inactivated FCS, and 10% Rat T-STIM (Collaborative Biomedical Products, Bedford, MA). To establish CTL lines, the generated CTLs were maintained by biweekly stimulation with the mitomycin C-treated I10(325v)-pulsed Neo and were termed LINE-IIIB(325D) cells. CTL clones were established from bulk CTL lines using a limiting dilution technique in 96-well U-bottomed microplates, as described previously (15).

CTL assay

The cytolytic activity of the CTL lines and clones was measured, as previously described (27), using a standard 5-h ⁵¹Cr-release assay with various ⁵¹Cr-labeled targets, as indicated in the figure legends.

Flow cytometric analysis

Flow cytometric analysis was performed to determine the surface molecule expression of the established CTL lines and clones using a FACScan analyzer (Becton Dickinson Immunocytometric Systems, Mountain View, CA). We harvested 5×10^5 cells, washed them twice with serum-free RPMI 1640, and then pelleted them. Fluorescein isothiocyanate-conjugated monoclonal antibodies were added to pellets, and they were then incubated for

30 min at 4°C. Then, the cells were washed three times and resuspended with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.1% sodium azide. Dead cells were gated out by forward and side scatter based on propidium iodide uptake. Ten thousand events were acquired for each sample and analyzed using Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

mRNA extraction, reverse transcription, and PCR amplification

Poly(A) tail-bearing mRNA was isolated from CTL clones using the Fast Track mRNA Isolation Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription and PCR amplification of purified mRNA were performed with a GeneAmp RNA PCR Kit (PE Biosystems, Foster City, CA). For the synthesis of T-cell receptor V β -specific cDNA, V β 7-specific primer (ACATCCCTAAAGGATACAGGG) and V β 8-specific primer (ATATCCCTGATGGGTACAAGG) were used in conjunction with C β primer (CCGATGGGAGCACACGAACCCTTAAG). For the synthesis of T-cell receptor V α -specific cDNA, V α 2-specific primer (AGCAATTCTGAAGTGCAGTTA), V α 3-specific primer (CAGCCCGA-TGCTCGCGTCACT), and V α 16-specific primer (ATGGACTGTGTGTA-TGAAAC) were used in conjunction with C α primer (ACTGGACCACA-GCCTCAGCGTC).

DNA sequence analysis

PCR products were separated by electrophoresis on a 1% of agarose gel and sliced bands were purified by EASYTRAP glass powder (Takara Bio., Siga, Japan). We then analyzed the purified DNA by a direct-sequence technique, using the ABI PRISM Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Biosystems), and we analyzed the sequences on the ABI PRISM 377XL DNA Sequencing System (PE Biosystems).

Molecular modeling

Because the actual 3D TCR structures for both V β 7 and V β 8.3 were unknown, we carried out comparative and homology modeling to elucidate the spatial relation between TCR β (V β 7 or V β 8.3) and P18-I10 or I10(325v). Comparative modeling predicts the 3D structure of a given protein sequence (target) primarily on the basis of its alignment to one or more proteins of a known 3D structure (templates). It is usually difficult to accurately determine the 3D structure from a protein sequence by theoretical procedures. However, because the 3D TCR β structure consists of a sandwich comprising a four-stranded antiparallel β -sheet and a three-stranded antiparallel β -sheet that are linked by a disulfide bond, and because the core portion was tightly bound by a hydrogen bond, the 3D structure of the TCR β , especially the core portion, could be reliably predicted.

Following the above procedure, the so-called threading or 3D template-matching method (28) could be implemented to select templates. For this procedure, we used LIBRA (29) (http://www.ddbj.nig.ac.jp/search/libra_i-e.html) software in which compatible structures of a target sequence are sought from the structural library chosen from the Protein Data Bank (PDB), and the target sequence and 3D profile are aligned by simple dynamic programming. According to the alignment, sequence remounts on the structure and its fitness are evaluated by the pseudoenergy potential. The scores are then sorted from the best-matched templates and shown along with their alignments. Based on the obtained alignments between the template and the target V β sequence, a 3D model is calculated by the MODELLER software (30–33), by which five 3D models were obtained from five templates used.

To examine the interaction between the calculated TCR and P18-I10, TCR/peptide/class I MHC complex (PDB code 1kj2) was used for two reasons. First, the 3D structure of the MHC part of 1kj2 is almost identical to that of MHC used in our experiment, which is also resolved (code 1bii) and shows a H-2D^d class I MHC molecule presenting the HIV-derived peptide

P18-I10 (RGPGRFVTI) (34). The amino acid sequence identity between 1kj2 and 1bii is ~90%. Second, 1kj2 is a TCR/peptide/MHC complex (5), whereas 1bii is only a peptide/MHC complex. In our 3D models, V β 7 or V β 8.3 was fitted to the TCR β position in 1kj2 and was drawn by Mol Feat software (FiatLux, Tokyo, Japan).

Quantum chemical calculation

To study the effect of electric charge on the TCR recognition response, we calculated both the electronic state of P18-I10 and any changes therein based on a single amino acid substitution (325V with 325T) within the P18-I10 using the PM5 molecular orbital method (MOPAC2002) (35). In this calculation, hydrogen atoms were first added to the PDB 3D structures, and then the net charges of all atoms in them were obtained, with the PDB 3D structure being maintained.

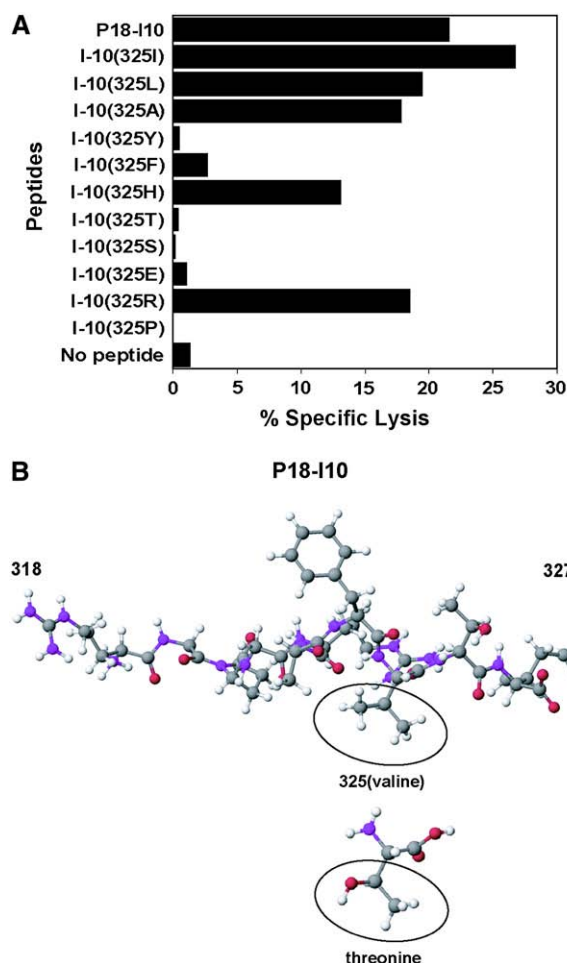


FIGURE 1 Effect of a single amino acid substitution at position 325 within P18-I10 on LINE-IIIb recognition. (A) Ten thousand of P18-I10-specific CTL line (LINE-IIIb cells) were added to 5000 ^{51}Cr -labeled BALB/c.3T3 fibroblast target cells in the presence of 3 μM of the substituted peptides at position 325 within P18-I10, as shown in Table 1. Standard errors of the means of triplicate cultures were <5% of the mean in each case. Results are representative of three independent experiments. (B) Ball-and-stick model of peptide P18-I10. The amino acid side chain containing the valine (V) at position 325 within P18-I10 and threonine (T) are shown in the circle. Carbon, oxygen, nitrogen, and hydrogen are shown in gray, red, blue, and white, respectively.

RESULTS

Effect of a single amino acid substitution at position 325 within P18-I10 on LINE-IIIb recognition

First, to see the effect of a single amino acid substitution at position 325 where epitope specificity for LINE-IIIb recognition is determined, a series of P18-I10-derived peptides shown in Table 1 have been synthesized. Similar to our previous findings (10), we confirmed that LINE-IIIb cross-reacted with an aliphatic residue, such as I, L, or A at position 325, in addition to reacting with the original residue V. Moreover, LINE-IIIb cross-reacted with positively charged residues such as R and H but not with the negatively charged E or the uncharged T, S, or P at position 325 (Fig. 1 A). Here, we should focus on the case of T, because the 3D structure of the side chain of T is very similar to that of V. When only one carbon atom of the side chain of V is exchanged for one oxygen atom (Fig. 1 B; red), the amino acid becomes T, except for the hydrogen atoms. According to the quantum chemical calculation using Hamiltonian PM5, the net charge of the carbon atom in V was about $-0.3e$, whereas that of the oxygen atom in T is about $-0.4e$; e represents the elementary charge. This may be the reason why the recognition of V at position 325 by LINE-IIIb was dramatically changed by substitution with T. These results indicate that the charges of the amino acids within the epitope may affect the interaction between TCRs and peptide/MHC complexes.

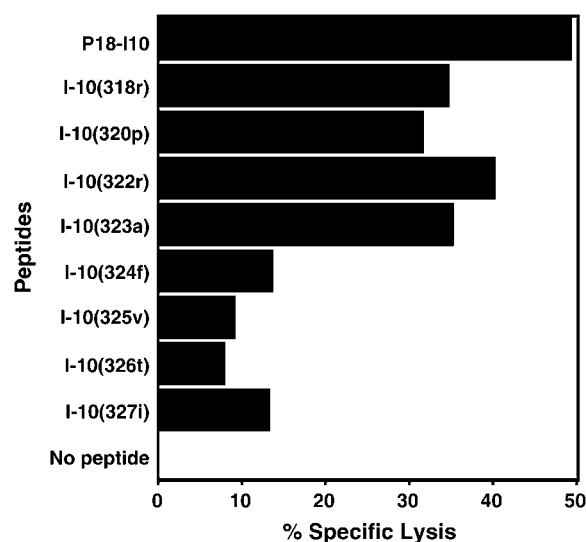


FIGURE 2 Effect of a single amino acid substitution with D-amino acid within an epitope peptide, P18-I10, on LINE-IIIb recognition. Ten thousand LINE-IIIb cells were added to 5000 ^{51}Cr -labeled BALB/c.3T3 fibroblast target cells in the presence of 3 μM of the substituted peptides with a single amino acid substitution by D-amino acid at each corresponding site (represented by lower-case letters in Table 1). Standard errors of the means of triplicate cultures were <5% of the mean in each case. Each experiment was performed at least three times.

Effect of a single amino acid substitution with D-amino acid within an epitope peptide, P18-I10, on LINE-IIIIB recognition

Second, to examine the effect of amino acid substitution with the same weight and charged D-amino acid on LINE-IIIIB recognition, a series of P18-I10-derived peptides with a single amino acid substitution by D-amino acid at each corresponding site represented by small letters in Table 1 has also been synthesized. When the corresponding D-amino acid was substituted at position 324, 325, 326, or 327, the cytotoxic activity of LINE-IIIIB was markedly reduced when compared with other substituted peptides (Fig. 2). This finding suggests that CTL-TCRs can strictly recognize each amino acid within the C-terminal half of the epitope peptide, including at position 325, which is critical for determining epitope specificity, and this is in contrast to their poor ability to recognize each amino acid within the N-terminal half.

Induction of CTL line and clones specific for L-valine or D-valine at position 325 within P18-I10

Then, to study the detailed molecular interactions in determining T-cell specificity, we attempted to generate a CTL line specific for I10(325v) having a single D-type amino acid substitution in P18-I10 at position 325 using immunization of BALB/c mice with syngeneic splenic dendritic cells

pulsed with the peptide (23). The I10(325v)-specific CTL line, LINE-IIIIB(325D), was successfully established. Although LINE-IIIIB(325D) showed some cross-reactivity to P18-I10 in a dose-dependent manner, it was highly specific for I10(325v) (Fig. 3 A). Similarly, LINE-IIIIB had some cross-reactivity to I10(325v)-sensitized targets (Fig. 3 B).

Using limiting dilution techniques described elsewhere (17), we successfully established two clones, IIE11(D) and IIA4(D), predominantly specific for I10(325v) but not for P18-I10 from LINE-IIIIB(325D) cells (Fig. 3 C) as well as two P18-I10-specific CTL clones, IIH7(L) and IB9(L), from the LINE-IIIIB cells (Fig. 3 D). Thus, we had established four highly specific clones, two of which were specific for the D-type of valine (v), and the other two for the L-type of valine (V) at position 325 within P18-I10.

Specificity and characterization of the established CTL clones

We further examined the fine specificities of the CTL clones using a series of substituted peptides, each with a single amino acid substitution of either the L- or D-type having an aliphatic or aromatic structure at position 325 in P18-I10 (Table 1).

Among the D-specific clones, IIE11(D) did not cross-react with I10(325I) at all and was strictly specific for the D-type

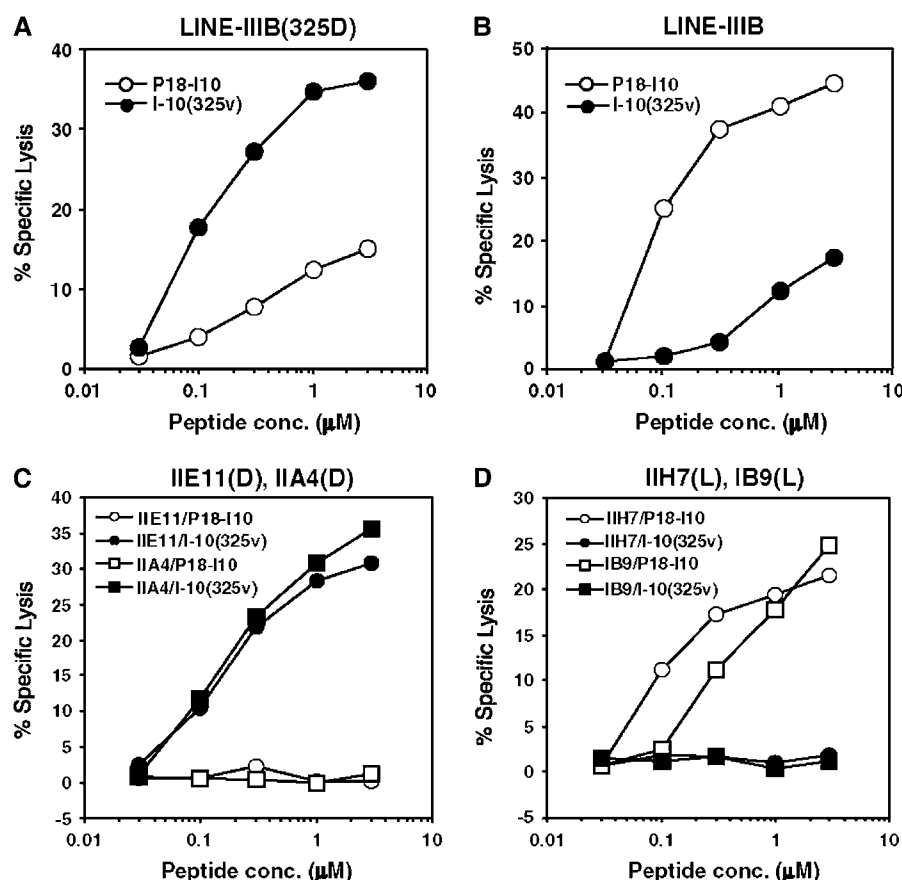


FIGURE 3 Comparison of specificity of the newly established CTL lines and clones against two peptides, peptide P18-I10 and I10(325v). We examined the cytolytic activity of the following distinct CTL lines and clones using a 5-h ^{51}Cr -release assay. To test the peptide specificity, effector cells and ^{51}Cr -labeled BALB/c.3T3 fibroblast targets (E/T ratio was 10:1) were incubated in the presence of various concentrations of either P18-I10 or I10(325v). (A) LINE-IIIIB(325D) specific for I10(325v) was used for effector cells. (B) LINE-IIIIB specific for P18-I10 was used for effector cells. (C) The CTL clones, IIE11(D) and IIA4(D), derived from LINE-IIIIB(325D) were used as effector cells. (D) The CTL clones, IIH7(L) and IB9(L), from LINE-IIIIB were used as effector cells. Standard errors of the means of triplicate cultures were $<5\%$ of the mean in each case. Each experiment was performed at least three times.

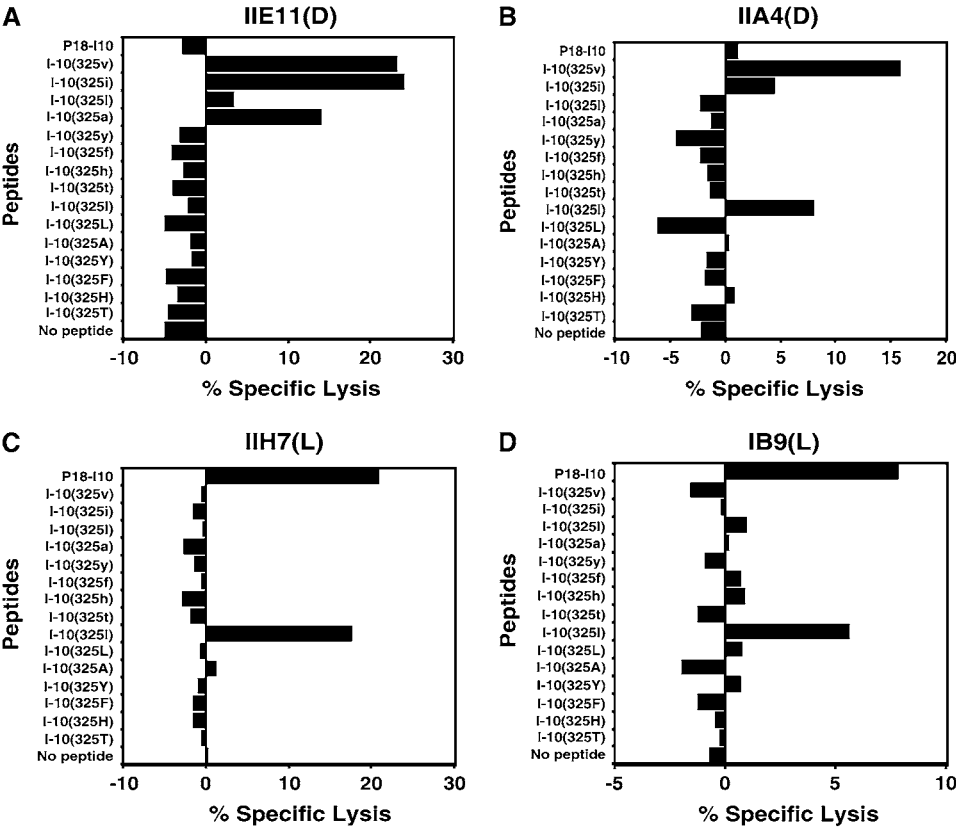


FIGURE 4 Effects of a single amino acid substitution at position 325 within P18-I10 on target sensitization for CTL recognition. We measured the cytolytic activity of the CTL lines and clones using a 5-h ^{51}Cr -release assay. For the analysis of fine peptide specificity, effector cells and ^{51}Cr -labeled BALB/c.3T3 fibroblast targets were mixed with 10 μM of each substituted peptide. The following four clones were used as effectors (E/T ratio was 20:1): (A) clone IIE11(D), (B) clone IIA4(D), (C) clone IIH7(L), and (D) clone IB9(L). Standard errors of the means of triplicate cultures were $<5\%$ of the mean in each case. Each experiment was performed at least three times.

substitution such as I10(325i) and I10(325a) as well as for the original I10(325v) (Fig. 4 A), whereas IIA4(D) showed a weak cross-reactivity for both types of isoleucine, I10(325I) and I10(325i), as well as for the original I10(325v) (Fig. 4 B). In addition, both of the L-type-specific CTL clones, IIH7(L) and IB9(L), cross-reacted with I10(325I) as well as with the original P18-I10 (Fig. 4, C and D). These findings indicate that the CTL clones can specifically distinguish the optical isomers at position 325.

Because P18-I10-specific CTL and their clones are CD8^+ , D^d -class I MHC molecule-restricted conventional $\alpha\beta\text{T}$ lymphocytes, we next confirmed the surface molecules and MHC-restriction of the I10(325v)-specific line and clones. LINE-IIIB(325D) and two clones, IIE11(D) and IIA4(D), were all CD3^+ , CD4^- , CD8^+ , and $\text{TCR}\alpha\beta^+$ by FACS analysis (data not shown), and they did not show any cytotoxicity against NK-sensitive YAC-1 cells (data not shown). Moreover, using three L cell (H-2^k) transfectants expressing the class I MHC of the d-haplotype, T4.8.3 (D^d), T1.1.1 (L^d), and B4III2 (K^d) (25,26), we confirmed that the I10(325v)-specific CTL line and clones were restricted by the D^d class I MHC molecule (data not shown).

TCR-sequences of the established clones

Taken together, the two groups of CTL clones with high specificity to the substituted P18-I10-derived peptides, having

either the L-type of valine (V) or D-type of valine (v) at position 325, expressed both CD8 and $\alpha\beta$ TCRs, and were restricted by the same class I MHC molecule, D^d . Therefore, taking advantage of the unique combinations of CTL clones, we attempted to perform a precise analysis of the interaction between the TCRs of those clones and the amino acid at

TCR α chain				
CTL clones	V α	N	J α	
IIE11(D)	CAMR	EAD	SNYQLIWGSGTKLIKPD	V α 16-J α 18BBM142
IIA4(D)	CAMR	EAD	SNYQLIWGSGTKLIKPD	V α 16-J α 18BBM142
IIH7(L)	CALS	ED	SNYQLIWGSGTKLIKPD	V α 3-J α 18BBM142
IB9(L)	CAAS	D	SNYQLIWGSGTKLIKPD	V α 2-J α 18BBM142

TCR β chain				
CTL clones	V β	N-D-N	J β	
IIE11(D)	CASS	DWGGG	TGQLYFEGGSKLTVL	V β 8.3-J β 2.2
IIA4(D)	CASS	DWGGG	TGQLYFEGGSKLTVL	V β 8.3-J β 2.2
IIH7(L)	CASS	LGVT	EVFFGKGTRLTVV	V β 7-J β 1.1
IB9(L)	CASS	LGVT	EVFFGKGTRLTVV	V β 7-J β 1.1

FIGURE 5 Nucleotide and amino acid sequences that form the V(D)J region of the TCR α and β chain from the established CTL clones. We analyzed the amino acid sequences of TCRs in two groups of CTL clones with high specificity to substituted P18-I10-derived peptides, having either the D-type of valine (v) (IIE11(D) and IIA4(D)) or the L-type of valine (V) at position 325 (IIH7(L) and IB9(L)) restricted by the same class I MHC molecules, D^d .

position 325 within epitope to determine the specificity by comparing their TCR sequences.

To investigate the actual amino acid sequences of the TCRs in the CTL clones, we extracted their mRNA and analyzed the nucleotide sequences of the α - and β -chain transcripts after a cDNA synthesis and PCR amplifications. The $V\alpha$ usage of the I10(325v)-specific clones, IIE11(D) and IIA4(D), were both $V\alpha 16$ (36), whereas that of the P18-I10-specific clones, IIH7(L) and IB9(L), were $V\alpha 3$ (37) and $V\alpha 2$ (BLASTN Accession U88296), respectively (Fig. 5). It should be noted that all of the four distinct clones used the same uncommon $J\alpha$ gene segment, 18BBM142, determined from a murine alloreactive T-cell hybridoma specific for I-A^{bm12} (38). In contrast, the $V\beta$ usage of the 325(v)-specific clones, IIE11(D) and IIA4(D), was $V\beta 8.3$ (39) with the $J\beta$ 2.2 segment (40) bearing the same CDR3, "DWGGS," whereas the $V\beta$ usage of the 325(L)-specific clones, IIH7 and IB9, was $V\beta 7$ with the $J\beta 1.1$ segment (41) encoding a distinct sequence, "LGYT" and "LGVT," respectively, in the CDR3

flap (Fig. 5). These results strongly indicate that the TCR β chains of those clones may be responsible for the specificity of the epitope.

Identification of the interaction site between the TCRs and a critical amino acid at position 325 to determine epitope specificity by molecular modeling analysis

Based on the above findings, we then studied the site that determines the epitope specificity of the TCR β chain in the CTL clones using a 3D molecular modeling analysis (see Materials and Methods). We used the LIBRA software (29,42) to select five compatible templates with excellent Standardized Scores (SD value) for both $V\beta 7$ and $V\beta 8.3$ (Fig. 6 A). Fig. 6 B shows the alignment of the five most suitable templates for each TCR.

We then used the MODELLER software (30,31,33) to model the 3D structure of the TCR. First, to confirm the

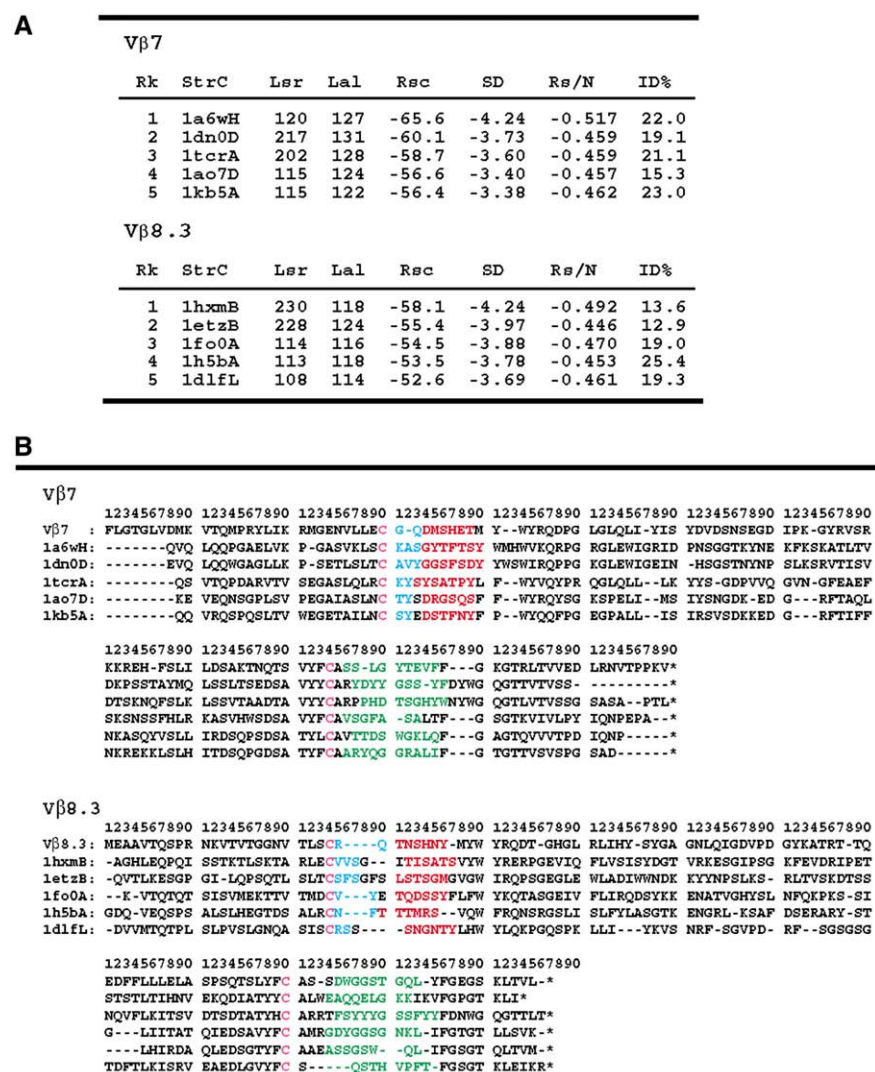


FIGURE 6 (A) Compatible templates with excellent Standardized Scores (SD value) for both $V\beta 7$ and $V\beta 8.3$ selected from the LIBRA software. Bold abbreviations are defined as follows: Rk, rank position; StrC, structural code of PDB (the last character is a subunit name); Lsr, length of the structural template; Lal, length of the aligned region; Rsc, raw score of the structural template; SD, standardized score; Rs/N, raw score (Rsc) normalized by the alignment length (Lal); ID%, sequence identity. (B) Sequence alignment of TCR $V\beta 7$, $V\beta 8.3$, and each of the five template proteins (1a6wH, 1dn0D, 1tcrA, 1ao7D, 1kb5A for $V\beta 7$ and 1hxmB, 1etzB, 1fo0A, 1h5bA, 1dlfL for $V\beta 8.3$) obtained from the LIBRA software. In this figure, the amino acid sequences of CDR1 and CDR3 from TCR $V\beta 7$ or $V\beta 8.3$ are drawn in red and green, respectively. In 10 template proteins, the corresponding portion of the CDR1 and CDR3 regions are also drawn in red and green, respectively. Conserved cysteine residues upstream of CDR1 and CDR3 are drawn in magenta. Two or three amino acids between the conserved cysteine residues and CDR1 are drawn in blue (see Discussion).

accuracy of our molecular modeling method, we predicted the 3D structure of a previously analyzed protein using MODELLER and then compared it with the experimental 3D structure obtained from x-ray crystallographic analysis for the same protein. We selected two proteins (PDB cord 1dn0D and 1a6wH) having high scores in Fig. 6 A and then calculated the 3D structure of 1dn0D using 1a6wH as a template. As shown in Fig. 7, the 3D structure of 1dn0D obtained from MODELLER was similar to that obtained experimentally, and their core regions were nearly identical. These results suggest that our molecular modeling method could be useful for the structural analysis of unknown TCRs.

We next predicted the 3D structures of TCR V β 7 and V β 8.3 and analyzed their interactions with P18-I10. The obtained 3D structures for V β 7 using the five selected templates were quite similar (Fig. 8, A–D) and could be confirmed by rotating the calculated structures from various angles (data not shown). In addition, each obtained 3D structure for V β 7 was fitted to the TCR β part in 1kj2 to analyze the interaction with the P18-I10 peptide. Although both the CDR1 (blue) and CDR3 (red) in V β 7 appeared to interact with the C-terminal half of P18-I10, the critical site

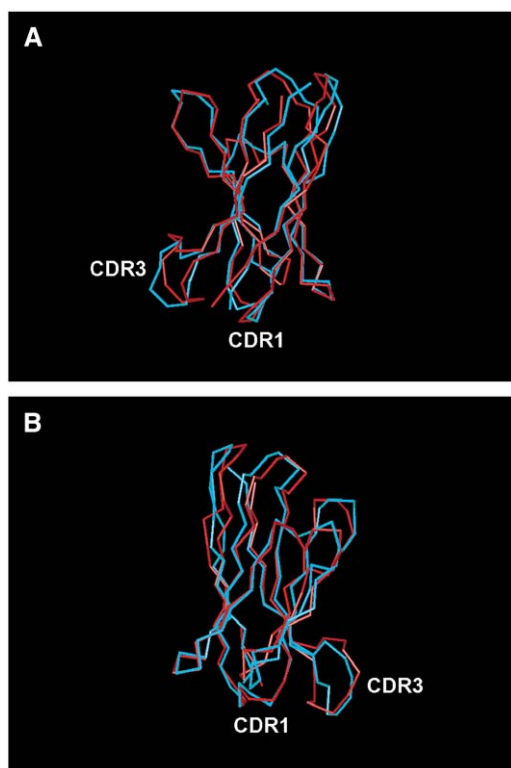


FIGURE 7 Comparison between the predicted 3D structure for 1dn0D by the MODELLER software and that of the same protein registered in PDB. The 3D structure of the protein (PDB cord 1dn0D) was predicted by the MODELLER, a molecular modeling software, using a protein (e.g., PDB cord 1a6wH) as a template. The predicted 3D structure for 1dn0D is drawn in blue, and the PDB 3D structure is drawn in red. Their backbone representations in the molecular modeling are drawn from rotated two distinct views (A and B).

for determining epitope specificity (325V, *bright green*) within P18-I10 was found to be more closely associated with the canonical free bottom portion of CDR1 and not with the CDR3 loop (Fig. 8, A and B): the CDR3 loop is too far from 325V. However, when the 325V was substituted with 325v (*bright red*), the CDR1 loop of V β 7 might come in contact with the 325v (Fig. 8, C and D), which seemed to induce conformational interference between the TCR and I10(325v), and thus, the H-2D^d-restricted peptide I10(325v) would not be recognized by the P18-I10-specific clones, IIH7(L) and IB9(L). These results are consistent with our experimental results and indicate that the CDR1 loop of TCR V β 7 should be the key site for determining P18-I10 specificity in the interaction with the L-valine at position 325.

Similarly, the molecular modeling for V β 8.3 was performed using another five of the most suitable templates shown in Fig. 6 A; each of these actual sequences is shown in Fig. 6 B. The calculated 3D structures of V β 8.3 based on the five templates had almost the same features (Fig. 8, E–H) as seen in the case of V β 7. In contrast to the case of V β 7, 325v (*bright red*) seemed to be associated with the free bottom portion of the CDR1 loop of V β 8.3 but not with the CDR3 loop of V β 8.3 (Fig. 8, E and F). However, the distance between the 325V (*bright green*) and the CDR1 loop in the case of V β 8.3 appeared to be greater than that in the case of V β 7 (Fig. 8, G and H), which can make a good contact with the 325V in P18-I10.

To substantiate the interaction between valine at position 325 and TCR-CDR1, the distance between terminal atoms in the side chain of amino acids within the epitope peptide and atoms in the main chain of the nearest portion within the TCR was calculated. As shown in Table 2, first to confirm the reliability of our molecular modeling, the distance between the two terminal atoms (OD1 and OD2) in the side chain of the seventh amino acid, aspartic acid (D), within epitope peptide pKB1(aa: KVTIFIDL) and the nearest portion in the TCR from OD1 or OD2 was determined using an already reported TCR/peptide/MHC complex, 1kj2 (PDBcode) obtained from x-ray crystallographic analysis (5). Based on the above observation, the distance between the two terminal atoms (CG1 and CG2) in the side chain of 325V or 325v and their surrounding atoms in the main chain of the obtained TCRs, V β 7 and V β 8.3, calculated (Tables 3 and 4) and compared with that of 1kj2. The results indicate that the distance between 325V and CDR1 in V β 7 is similar to the distance in the case of 1kj2, whereas the distance between 325v and CDR1 in V β 7 is too small, which may induce conformational interference between them. In contrast, the distance between 325v and CDR1 in V β 8.3 is similar to 1kj2, but the distance between the 325V and the CDR1 in V β 8.3 appeared to be too far from that of 1kj2. These results again agreed with our experimental results that V β 8.3 recognized the epitope I10(325v) having D-valine at position 325 via CDR1 but did not recognize the P18-I10 containing L-valine at that position.

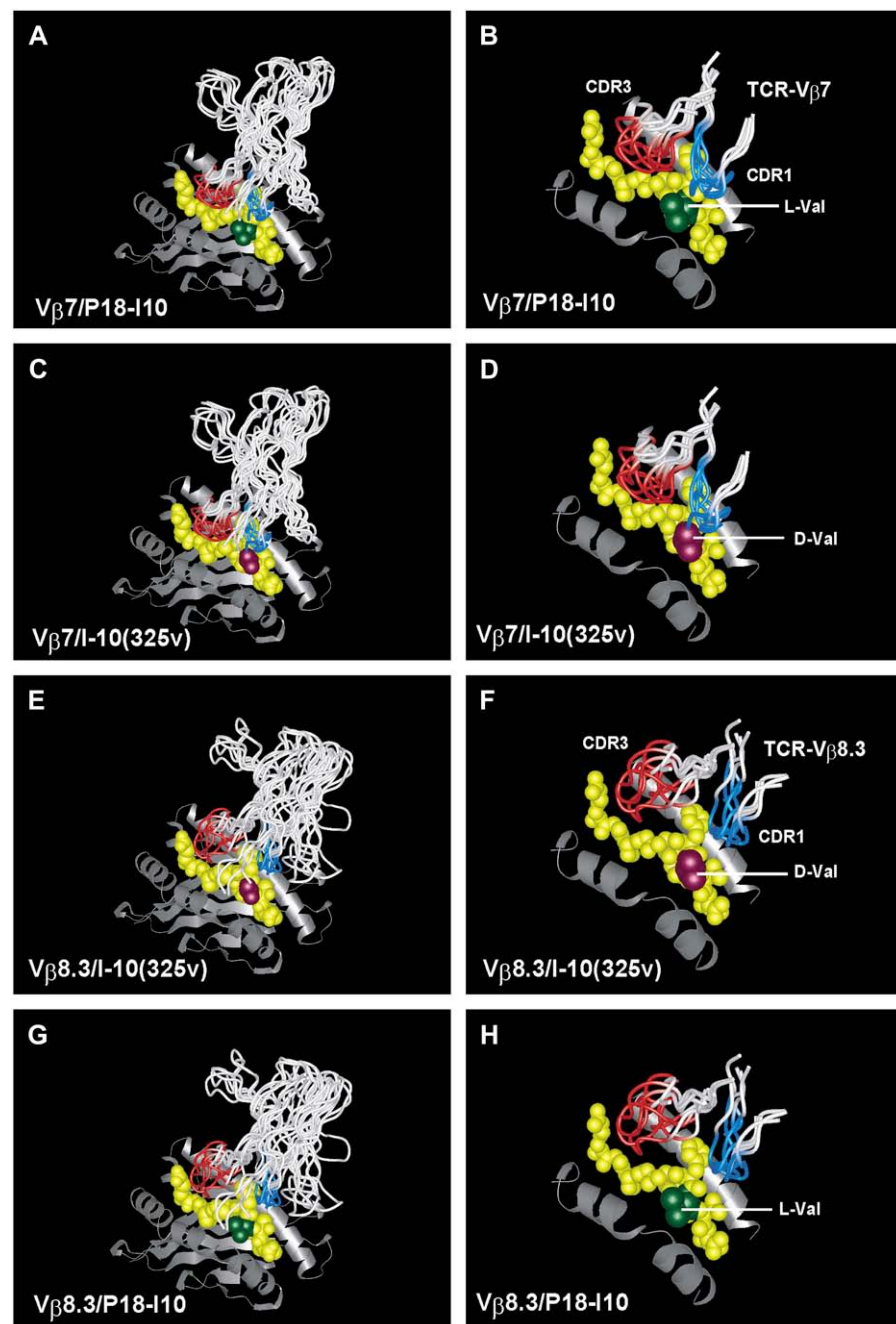


FIGURE 8 3D structures representing the interaction between TCR V β 7 (V β 8.3) and P18-I10/H-2D^d Complex or the interaction between TCR V β 7 (V β 8.3) and I10(325v)/H-2D^d complex. (A–H) The ternary complex of TCR V β 7 or V β 8.3 onto either P18-I10 or I10(325v) bound to the H-2D^d class I MHC molecules were illustrated by computer-based molecular modeling (see Materials and Methods). Horizontal gray backbone represents the H-2D^d class I MHC molecule, and the yellow ball format indicates the D^d-bound epitope peptide P18-I10. Vertical overlapping white backbones indicate either TCR V β 7 or V β 8.3. In these figures, V β 7 and V β 8.3 made from five distinct template proteins were overlaid in one figure. The CDR1 and CDR3 loops from both TCR V β chains are drawn in cyan and red, respectively. The L-type of valine (V) and the D-type of valine (v) at position 325, where epitope specificity appears to be determined, are shown in bright green and bright red, respectively. The 3D-structures of the (A) V β 7/H-2D^d/P18-I10, (B) enlarged figure of A, (C) V β 7/H-2D^d/I10(325v), (D) enlarged figure of C, (E) V β 8.3/H-2D^d/I10(325v), (F) enlarged figure of E, (G) V β 8.3/H-2D^d/P18-I10, and (H) enlarged figure of G are shown in eight independent panels.

Therefore, CDR1, but not the CDR3 loop, in V β 7 or V β 8.3 seems to play an important role in the recognition of each specific epitope peptide. Moreover, amino acids at positions 26 to 31: DMSHET within the CDR1 of V β 7, or TNSHNY within the CDR1 of V β 8.3, appear to interact with the critical amino acid at position 325 to determine the epitope specificity.

DISCUSSION

In our previous study, we found that the amino acids at positions 322R and 324F were critical for D^d binding, and

those at position 325V were essential for interacting with TCRs (8,17). Also, the C-terminus 327I appears to be a key amino acid for D^d binding to form the D^d-binding motif (8,18). In this study, we demonstrated that the substitution of a positively charged 322R by 322r did not result in measurable changes in target sensitization, although in our previous study the substitution with uncharged alanine (A) completely eliminated the capacity to sensitize the targets (17), indicating that a positive electric charge must be critical for D^d binding at position 322, and that a reduction of charge in the amino acid might diminish the epitope potency for T-cell activation. As shown in Fig. 1 B, the side chains of V and T have the same 3D

TABLE 2 The distance between two terminal atoms in the side chain of the seventh amino acid, Asp (D) within epitope peptide and atoms in main chain of the nearest portion within the TCR

	N (S28)	C _α (S28)	C (S28)	O (S28)	N (Q29)	C _α (Q29)	C (Q29)	O (Q29)	N (Y30)	C _α (Y30)	C (Y30)	O (Y30)
OD1	10.86	9.55	8.37	7.24	8.76	7.98	7.60	6.99	8.27	8.39	8.29	9.37
OD2	8.83	7.48	6.40	5.24	6.97	6.44	6.21	5.91	6.76	7.08	6.84	7.88

Unit of the distance is Å. The values are calculated using 1kj2 (PDB code), which is TCR/peptide/ MHC complex structure obtained from x-ray crystallographic analysis. The OD1 or OD2 represents the terminal atom of the side chain in the seventh amino acid, aspartic acid (D), within epitope peptide pKB1(aa: KVITFIDL). The amino acids Ser 28 (S28), Gln 29 (Q29), and Tyr 30 (Y30) are the nearest portion within the TCR from the OD1 or OD2.

structure, except for the hydrogen atoms, if one terminal carbon atom of the side chain of V is exchanged for one oxygen atom. According to the quantum chemical calculation, the net charge of the carbon or the oxygen is $-0.3e$ or $-0.4e$, respectively. In addition, three hydrogen atoms, which have positive charges, are added to the carbon, whereas one hydrogen atom is added to the oxygen. Thus, T generates a greater negative net charge than V. The influence of this charge difference was evident when LINE-IIIB recognized the peptide. It is likely that T had a greater repulsive force against the oxygen atoms in the CDR1 loop compared with V. This repulsive force may account for the significant reduction in the recognition by LINE-IIIB after substitution with T. These findings indicate that the charge of an amino acid at a specific position within an epitope can affect its binding capacity to MHC molecules and/or the interaction with a TCR.

Although the method we applied here for modeling the TCR/peptide/class I MHC complex was not directly based

on crystallographic analysis, our computer-based molecular modeling was still accurate. Indeed, as demonstrated in Fig. 7, the predicted 3D structure for 1dn0D by MODELLER was similar to that determined experimentally, and the core regions appeared to be nearly identical. This reflects the fact that the 3D structures of the core regions of the TCR consist of a four-stranded antiparallel β -sheet and a three-stranded anti-parallel β -sheet linked by a disulfide bond. Although the predicted 3D structure of CDR3 seems slightly different from the experimental 3D structure of CDR3 because of the large loop, the predicted 3D structure of CDR1 appears almost the same as the experimental 3D structure. These results suggest that the TCR domain predicted by the present molecular modeling methods can become useful and reliable tools for the structural analysis of TCRs.

In these sorts of structural analyses, it should be acknowledged that a substitution of D-valine (v) for L-valine (V) would result in a conformational interference between the class I MHC molecule and the D-valine (v), which itself could decrease the recognition response by LINE-IIIB. However, as shown in Fig. 9, no conformational interference occurred in this study. Thus, I10(325v) would also be associated with the class I MHC molecule so that TCRs may recognize the epitope. Therefore, our findings suggest that the change in the recognition response by LINE-IIIB after the D-valine (v) substitution reflects the interaction with the TCR.

Our molecular modeling analysis demonstrated that the critical area of the TCR for interacting with 325V within P18-I10 was the peptide DMSHET, within the CDR1 of V β 7. In contrast, the substituted peptide with the D-type amino acid, I10(325v), was recognized by the peptide TNSHNY within the CDR1 of V β 8.3. Therefore, the CDR1 in the V β 7 or V β 8.3 might play an important role in recognizing the epitope P18-I10 or I10(325v), respectively, and, in particular, the distance between CDR1 and the amino acid 325V or 325v within the peptide seemed to be essential for recognizing the epitope. Taken together, the results derived from our molecular modeling strategy appear to be consistent with the experimental results.

Although the epitope specificity created by TCRs has been reported to be dependent mainly on the amino acid sequences in the CDR3 regions for both the TCR α and β chains (43,44), crystallographic analyses on various TCR and peptide/MHC interactions have suggested that both the CDR1 and CDR3 in

TABLE 3 The distance between two terminal atoms in the side chain of 325V or 325v and atoms in main chain of the obtained TCR-CDR1 through molecular modeling

	V β 7	N(S)	C α (S)	C(S)	O(S)	N(H)	C α (H)	C(H)	O(H)
1a6wH CG1 (325V)	10.20	9.55	8.56	8.52	7.99	7.08	7.85	7.48	
CG2 (325V)	9.57	8.88	7.62	7.41	7.01	5.80	6.17	5.57	
1dn0D CG1 (325V)	10.03	9.43	8.73	8.11	9.12	8.90	9.30	9.33	
CG2 (325V)	9.40	8.79	7.77	6.99	8.06	7.58	7.65	7.48	
1trcA CG1 (325V)	7.69	6.38	6.49	7.45	5.86	6.44	7.84	8.34	
CG2 (325V)	6.63	5.41	5.09	5.76	4.51	4.80	6.29	6.97	
1ao7D CG1 (325V)	7.57	6.48	7.18	6.85	8.40	9.21	9.59	10.11	
CG2 (325V)	6.91	5.63	5.85	5.34	6.96	7.48	7.89	8.63	
1kb5A CG1 (325V)	7.51	8.02	7.58	6.44	8.67	8.81	9.71	10.44	
CG2 (325V)	6.77	7.06	6.21	4.99	7.08	6.92	7.89	8.79	
1a6wH CG1 (325v)	8.10	7.48	6.70	6.85	6.20	5.60	6.69	6.63	
CG2 (325v)	6.95	6.27	5.12	5.09	4.53	3.56	4.38	4.18	
1dn0D CG1 (325v)	7.94	7.34	6.88	6.41	7.39	7.47	8.10	8.42	
CG2 (325v)	6.79	6.16	5.28	4.59	5.73	5.59	5.91	6.14	
1trcA CG1 (325v)	6.51	5.12	5.35	6.50	4.59	5.40	6.57	6.84	
CG2 (325v)	5.01	3.61	3.26	4.28	2.35	3.02	4.34	4.78	
1ao7D CG1 (325v)	5.64	4.73	5.73	5.63	6.98	8.02	8.36	8.66	
CG2 (325v)	4.39	3.09	3.63	3.36	4.88	5.73	6.12	6.61	
1kb5A CG1 (325v)	5.60	6.21	6.12	5.18	7.37	7.86	8.74	9.24	
CG2 (325v)	4.21	4.55	4.02	2.91	5.16	5.49	6.49	7.13	

Unit of the distance is Å. The code 1a6wH, 1dn0D, 1trcA, 1ao7D, or 1kb5A represents each template protein to determine the structure of V β 7. CG1 and CG2 are two terminal atoms of side chain in 325V or 325v. The successive two amino acids, Ser and His, within CDR1 of V β 7 are the nearest portion for CG1 or CG2.

TABLE 4 The distance between two terminal atoms in the side chain of 325V or 325v and atoms in main chain of the obtained TCR-CDR1 through molecular modeling

V β 8.3	N(T)	C α (T)	C(T)	O(T)	N(N)	C α (N)	C(N)	O(N)	N(S)	C α (S)	C(S)	O(S)
1hxmBCG1 (325V)	13.34	12.22	11.94	12.32	11.47	11.47	10.62	9.61	11.19	10.74	11.80	12.32
CG2 (325V)	12.54	11.59	11.25	11.41	11.00	10.98	9.90	8.83	10.34	9.64	10.65	11.36
1etzBCG1 (325V)	13.77	13.45	12.19	11.26	12.24	11.31	9.99	8.95	10.16	9.20	9.41	10.25
CG2 (325V)	13.14	12.75	11.63	10.76	11.76	11.02	9.57	8.73	9.41	8.22	8.32	9.30
1fo0ACG1 (325V)	10.43	9.82	9.37	9.20	9.43	9.34	10.58	11.43	10.85	12.07	13.27	13.89
CG2 (325V)	9.41	8.58	8.29	8.45	8.18	8.25	9.66	10.47	10.12	11.47	12.47	13.09
1h5bACG1 (325V)	11.98	10.99	10.34	10.13	10.23	9.81	10.97	12.02	10.95	12.14	12.24	11.29
CG2 (325V)	11.18	10.43	9.87	9.95	9.52	9.16	10.18	11.13	10.20	11.29	11.10	10.02
1dlfLCG1 (325V)	12.46	11.35	10.28	9.72	10.18	9.27	10.19	11.40	9.77	10.82	11.61	11.18
CG2 (325V)	11.39	10.46	9.42	9.14	9.06	8.14	9.06	10.25	8.69	9.75	10.28	9.64
1hxmBCG1 (325v)	11.19	10.04	9.53	9.84	8.98	8.81	7.89	6.96	8.39	7.95	9.00	9.47
CG2 (325v)	10.21	9.16	8.72	8.95	8.37	8.32	7.31	6.25	7.84	7.33	8.47	9.12
1etzBCG1 (325v)	11.63	11.33	10.01	9.08	10.05	9.08	7.83	6.75	8.15	7.37	7.76	8.53
CG2 (325v)	10.57	10.17	9.00	8.11	9.13	8.38	6.95	6.11	6.87	5.78	6.11	7.09
1fo0ACG1 (325v)	8.55	8.16	7.68	7.31	7.99	7.98	9.10	9.82	9.44	10.57	11.78	12.26
CG2 (325v)	6.96	6.31	6.03	6.03	6.22	6.47	7.78	8.44	8.38	9.66	10.67	11.12
1h5bACG1 (325v)	9.98	8.91	8.17	7.84	8.17	7.74	8.99	10.05	9.05	10.33	10.63	9.80
CG2 (325v)	8.70	7.89	7.22	7.27	6.92	6.57	7.69	8.66	7.83	9.04	9.03	8.07
1dlfLCG1 (325v)	10.66	9.50	8.36	7.65	8.40	7.51	8.42	9.61	8.05	9.15	10.11	9.84
CG2 (325v)	9.11	8.13	6.97	6.57	6.70	5.75	6.71	7.91	6.41	7.59	8.30	7.82

Unit of the distance is Å. The code 1hxmB, 1etzB, 1fo0A, 1h5bA, or 1dlfL represents each template protein to determine the structure of V β 8.3. CG1 and CG2 are two terminal atoms of side chain in 325V or 325v. The successive three amino acids, Thr, Asn, and Ser, within CDR1 of V β 8.3 are the nearest portion for CG1 or CG2.

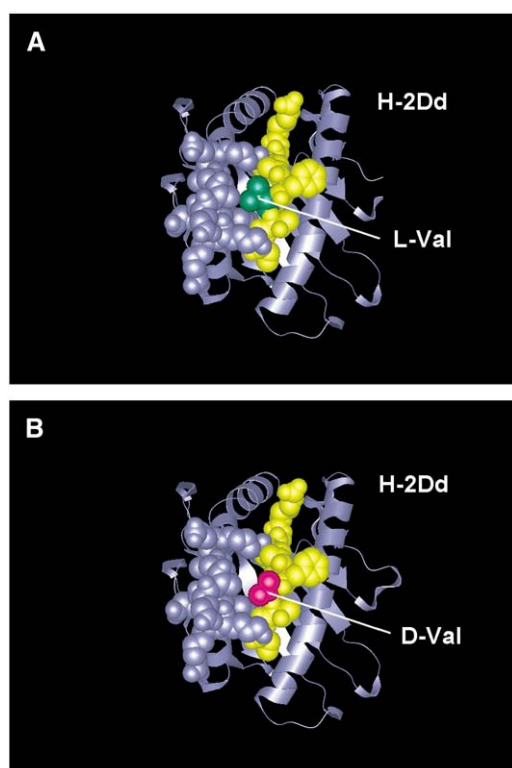


FIGURE 9 (A and B) 3D structures of P18-I10/H-2D^d complex and I10(325v)/H-2D^d complex. Gray ribbon and ball format represent the H-2D^d class I MHC molecule, and the yellow ball format indicates the D^d-bounded epitope peptide, P18-I10 or I10(325v). In the H-2D^d class I MHC molecule, only the positions for interaction with 325V are drawn in the ball format, for clarity. The L-type of valine (V) and the D-type of valine (v) at position 325 are shown in green and red, respectively. Radius of ball format indicates van der Waals radius.

the TCR α and TCR β chains might contact with the antigenic peptide/MHC complex (1,2,5,6), particularly with the carboxyl-terminal portion of the peptides (2). Moreover, recent reports have shown that the CDR1 in V β 10 participated in class I MHC molecule-mediated T-cell recognition (45,46). In these reports, significant alteration in the capacity to bind class I MHC molecules and in the ability to respond to the peptide/MHC complex was demonstrated when a single amino acid substitution was introduced into the CDR1 of TCR V β 10 by site-directed mutagenesis. Indeed, we have shown here that the CDR1 in the TCR β chains appeared to interact directly with the key amino acid for determining epitope specificity. Thus, if the most critical amino acid for determining the epitope specificity is located near the C-terminal portion of a peptide such as P18-I10, not only the CDR3 but also the CDR1 in the TCR β chain may be involved in determining antigen specificity.

It is also important to consider the role of the primary structures in TCR recognition. As demonstrated in Fig. 6 B, there are two cysteine (C) residues positioned upstream of CDR1 (*magenta*) and CDR3 (*magenta*) of both TCR V β 7 and V β 8.3. These residues bind each other via a disulfide bond and must be a basic structure of TCRs because they are conserved in most of the murine TCR sequences (47). The two or three amino acids (*blue*) between the conserved cysteine (C) residues and the CDR1 would be key amino acids in forming the 3D structure of CDR1, although they do not interact directly with the epitope peptide. If only these amino acids are exchanged for other amino acids, the 3D structure of CDR1 affecting the recognition of an amino acid at position 325 would change. Indeed, these amino acids are

highly variable for both TCR V β 7 and V β 8.3 (Fig. 6 B) and for various other murine TCR sequences (47), whereas the regions just after CDR1 are mostly conserved. These amino acids are likely to participate in the peptide recognition by creating small changes in the 3D structure of CDR1. In this regard, both CDR1 and the two or three amino acids between the cysteine (C) and CDR1 may play an important role in recognizing position 325 within P18-I10 or I10(325v).

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